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PRINCIPAL INVESTIGATOR: Yong-Dong Wang, Ph.D.

CONTRACTING ORGANIZATION: Health Research Incorporation
Buffalo, New York 14263

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Yong-Dong Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Health Research Incorporation
Buffalo, New York 14263

E-MAIL:
gnodgnoy@yahoo.com

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13. ABSTRACT *(Maximum 200 Words)*

Abnormal regulation of gene expression plays an important role in cancer. The first step in the regulation of gene expression requires the binding of transcription factor (TF) to its DNA response element in the gene promoter region. Therefore, interfering with TF-DNA complexes could be a powerful tool for blocking oncogene expression and elucidating how aberrant gene expression contributes to neoplastic phenotypes. The HER2/neu oncogene is amplified and transcriptionally upregulated in 25-30% of human breast cancers. This upregulation has been shown to depend on a highly conserved ETS binding site (EBS) and its upstream AP-2 binding site within the key regulatory region of the HER2/neu promoter. In this study, we investigated a new class of DNA minor groove binding ligands, hairpin pyrrole-imidazole polyamides, as potential TF-DNA inhibitor in gene expression. Several new polyamides were designed specifically targeted to these TF binding sites within HER2/neu promoter region. Polyamides represent a significant advancement in ligand design in that they can achieve a remarkable degree of sequence specificity and high affinity for predetermined DNA sequences. Our results indicate that the first generation of polyamides is potent inhibitors of TF-DNA complex formation and transcription under cell-free conditions.

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INTRODUCTION:

The ability to preferentially block gene expression by interfering with oncogenic transcription factor (TF)-DNA complexes could be a powerful tool for elucidating how aberrant gene expression contributes to neoplastic phenotypes (1). One approach to inhibit these complexes is to target DNA binding agents to the TF DNA binding site (2, 3). This study investigates a new class of DNA minor groove binding ligands, pyrrole-imidazole polyamides, as transcription inhibitors targeted at TF promoter elements. Ets protein ESX and AP-2 DNA binding domains contained within the regulatory region of the HER2/neu proximal promoter were chosen as the targets. The protein binding to the HER2/*neu* gene promoter results in a deregulation and overexpression of this growth factor receptor proto-oncogene that is linked to human breast cancer (4, 5). Sequence specific DNA minor groove binding polyamides were synthesized that bind with high affinity ($K_a \leq 10^{-10}$ M^{-1}) to ESX or AP-2 DNA binding sites (6-8). As measured by electrophoretic mobility shift assay (EMSA), polyamides binding to these sites were one to two orders of magnitude more effective than distamycin at inhibiting formation of complexes between the purified proteins and the HER2/*neu* promoter probes. The polyamides also effectively inhibit the HER2/*neu* promoter-driven transcription measured in vitro with nuclear extract from the HER2/*neu*-overexpressing human breast cancer cell line, SKBR-3. However, the examination of these polyamides in SKBR-3 cell revealed a limited ability to interfere with cellular gene expression based upon Northern analysis of Her2/neu mRNA. While studying other possible cellular activities of the polyamides, it was found that the polyamides inhibited the cellular uptake of uridine. To enhance the cellular activity of the polyamides a number of analogs were created with the idea of improving their activity in cell. No significant improvement was detected in both cell-free and cellular condition. Further modifications of these compounds are planned.

BODY:

Pyrrole and imidazole containing polyamides (PA) represent a new class of synthetic DNA binding ligands with remarkably high affinity and sequence-specificity. The code for their sequence-specific DNA recognition is based on a side-by-side pairing of the heterocyclic amino acid units within the minor groove of DNA. In this project, the activities of two polyamides, PA-2 and PA-10, were first tested both in cell-free and in whole cell system. PA-2 was designed to bind immediately downstream of the ESX core binding site while PA-10 was designed to bind the AP-2 binding site of the Her2/neu promoter.

The quantitative footprint titration analysis showed that PA-2 and PA-10 bind to their target sites with equilibrium association constant of $1.4x10^{10}$ M^{-1} and $8.7x10^{10}$ M^{-1} respectively. In this study, electrophoretic mobility shift assay was used to test the ability of each drug to inhibit its target binding site within the Her2/neu promoter. Incubation of PA-2 or PA-10 with a DNA oligonucleotide containing the binding site from Her2/neu promoter followed by the addition of ESX or AP-2 protein resulted in a concentration-dependent inhibition of transcription factor-DNA complex formation. Ten nM of PA-2 inhibited complex formation up to 95 % while 1 nM resulted in a detectable decrease in complex formation. Eight nM of PA-10 inhibited complex formation 95% while as little as 0.5 nM resulted in a detectable decrease in complex formation. The result showed that 2.2 nM of PA-2 and 1.2 nM of PA-10 are required to inhibit complex formation by 50%. The use of combination of PA-2 and PA-10, which target different locations on the promoter shows additive effect.

To determine whether the effects of drugs on transcription factor-DNA complex formation resulted in an ability to influence biological function, *in vitro* transcription assays were performed. The plasmid DNA containing the Her2/neu promoter was used as a template and SKBR-3 nuclear extracts as transcription machinery, resulting in a 760 base transcript. Drugs were incubated with DNA template prior to the addition of nuclear extracts and nucleotides. The result demonstrates the ability of drugs to block synthesis of the 760 base transcript in a concentration-dependent manner. For example, 0.5 μM PA-10 inhibited transcript synthesis by 95% while 0.1 μM blocked the transcript less than 30% compared with the untreated control. Drug concentrations of 2 μM for distamycin, 0.5 μM for PA-2, and 0.2 μM for PA-10 were required to inhibit transcription by 50%. There was some evidence of the production of partial transcripts when higher drug concentrations were used. Comparison of the abilities of the polyamides with that of distamycin to inhibit transcript synthesis reveals that the potency of former compounds are about one order of magnitude greater. The very high affinity constant of polyamides likely contributes to the difference in activity.

Since polyamides show strong activities in cell-free conditions, we next evaluate the effectiveness of polyamides as Her2/neu transcription inhibitions in the whole cell system. Northern analysis and RNA synthesis assays were performed. AP-2, AP-10 along with several other drugs were tested in the Northern analysis. Treatment with 50 μM of

distamycin for 48 hours decreases the Her2/neu mRNA of SKBR-3 cells by 50% while the mRNA of GAPDH is probed at the same time as comparison. However, no significant change of Her2/neu mRNA was detected after up to 20 μM polyamide treatment. Meanwhile, the cytotoxicity assay failed to detect any cellular activity of polyamides. The result in our lab also strongly indicated that majority of the polyamide was located in the nucleus. It is unclear why the polyamides are located in the nucleus but do not show any functional activity.

In the process of the RNA synthesis assays, we have developed a more sensitive condition to detect the drug effects. When the traditional method for this assay was applied, the result showed that some drugs including distamycin and polyamides not only inhibit uridine incorporation, but also decrease uridine uptake at a comparable level. This observation makes it difficult to study the effect of RNA synthesis by using the uridine incorporation assay. The separation of the effects for uridine uptake from incorporation was greatly improved by adding 25 μM of cold uridine in the medium. The high uridine concentration at a saturated level for the uridine uptake helps to eliminate the drug effect on the uridine uptake.

There is a detectable polyamide cellular activity when nucleoside uptake assay was performed. Out of fifteen polyamides tested, nine of them inhibit nucleoside uptake, while six of them do not. When the chemical structures between these two groups were compared, it was found that all polyamides with internal β-alanin such as PA-10 don't inhibit nucleoside uptake, while polyamides without internal β-alanin such as PA-2 inhibit nucleoside uptake. This observation is interesting, but at this point we are not sure how this might relate to the project, since the biological effect of inhibition is still unclear.

To understand factors in cellular environment that might interfere the polyamide-DNA binding, we take advantage of a fluorescent polyamide, PA-22. By using the fluorescent compound, we can detect the polyamide-DNA complex directly on an agarose gel. The fluorescent signal of polyamide-DNA complex is polyamide and DNA dose-dependent, and is competed by the parent polyamide, PA-2. This simple method allowed us to easily test the factors that interfere polyamide-DNA binding under cell-free condition. Factors that do not interfere polyamide-DNA binding include temperature (4°C - 70°C), pH (4 - 9.5), salt (up to 1M Na^+, 0.1M K^+, 10mM Ca^+), tRNA (up to 5μg/μl), BSA (up to 1%), and some cellular component such as polysome or nuclear extract. Factors that interfere polyamide-DNA binding include some detergents such as sarkosyl and SDS. These results will guide us to understand the basic pharmacological aspects of polyamide-DNA binding.

KEY RESEARCH ACCOMPLISHMENTS:

1. Sequence specific DNA minor groove binding polyamides bind with high affinity to ESX or AP-2 DNA binding sites in the HER2/neu promoter region.

2. As measured by mobility shift assay, polyamides inhibited formation of complexes between transcription factors and their DNA binding sites within the HER2/neu promoter region.

3. The polyamides effectively inhibit the HER2/neu promoter-driven transcription by using nuclear extract from the HER2/neu-overexpressing human breast cancer cell line, SKBR-3.

4. These polyamides showed a limited cellular activity based upon Northern analysis of HER2/neu mRNA or cytotoxicity assay.

5. Polyamides having the structure without internal β-alanine inhibit the cellular nucleoside uptake.

6. We conclude that polyamides have the potential to be designed specifically to block cellular proteins that are implicated in human breast cancers.

7. This study provides clues for future modifications of polyamide, one of the active compounds in cancer research.

REPORTABLE OUTCOMES:

Chiang, S.-Y., Burli, R., Benz, C.C., Gawron, L., Scott, G.K., Dervan, P.B. and Beerman, T.A. Targeting the Ets Binding Site of the Her2/neu Promoter with Polyamide in Cell-free System. (2000) J. Biol. Chem. 275, 24246-24254

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CONCLUSIONS:

We conclude that the first generation of polyamide are potent inhibitors of TF-DNA complex formation and transcription under cell-free conditions and that polyamides have the potential to be designed specifically to block cellular proteins that are implicated in human breast cancers. The information provided in this study can be utilized to improve polyamide specificity and effectiveness as selective transcription inhibitors. Modification of these compounds is under investigation to enhance their cellular activities.

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Targeting the Ets Binding Site of the HER2/*neu* Promoter with Pyrrole-Imidazole Polyamides*

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Shu-Yuan Chiang‡, Roland W. Bürli§¶, Chris C. Benz‖, Loretta Gawron‡, Gary K. Scott‖, Peter B. Dervan¶, and Terry A. Beerman‡§§**

From the ‡Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263, ¶Division of Chemistry and Chemical Engineering and Beckman Institute, California Institute of Technology, Pasadena, California 91125, and the ‖Department of Medicine, University of California, San Francisco, San Francisco, California 94143

Three DNA binding polyamides (1–3) were synthesized that bind with high affinity ($K_a = 8.7 \cdot 10^9$ M^{-1} to $1.4 \cdot 10^{10}$ M^{-1}) to two 7-base pair sequences overlapping the Ets DNA binding site (EBS; GAGGAA) within the regulatory region of the HER2/*neu* proximal promoter. As measured by electrophoretic mobility shift assay, polyamides binding to flanking elements upstream (1) or downstream (2 and 3) of the EBS were one to two orders of magnitude more effective than the natural product distamycin at inhibiting formation of complexes between the purified EBS protein, epithelial restricted with serine box (ESX), and the HER2/*neu* promoter probe. One polyamide, 2, completely blocked Ets-DNA complex formation at 10 nM ligand concentration, whereas formation of activator protein-2-DNA complexes was unaffected at the activator protein-2 binding site immediately upstream of the HER2/*neu* EBS, even at 100 nM ligand concentration. At equilibrium, polyamide 1 was equally effective at inhibiting Ets/DNA binding when added before or after *in vitro* formation of protein-promoter complexes, demonstrating its utility to disrupt endogenous Ets-mediated HER2/*neu* preinitiation complexes. Polyamide 2, the most potent inhibitor of Ets-DNA complex formation by electrophoretic mobility shift assay, was also the most effective inhibitor of HER2/*neu* promoter-driven transcription measured in a cell-free system using nuclear extract from an ESX- and HER2/*neu*-overexpressing human breast cancer cell line, SKBR-3.

Abnormal regulation of gene expression plays an important role in cancer (1, 2). The first step in the regulation of gene expression requires transcription factor (TF)[1] binding to its cognate DNA response element in the gene promoter region (3–5). The ability to preferentially block gene expression by interfering with TF-DNA complexes could be a powerful tool for elucidating how aberrant gene expression contributes to neoplastic phenotypes.

One strategy for developing gene-specific transcriptional inhibitors is to target DNA binding ligands to the cognate DNA response element of a crucial, promoter-regulating TF (6–8). A number of DNA binding natural products and their analogs, which interfere with the binding of TFs to their promoter response elements, are potent inhibitors of gene expression (9–14). Mithramycin, a G,C-specific DNA minor groove binder, inhibits c-Myc expression driven by its G,C-rich P1 promoter (9, 10). Similarly, small molecules such as the DNA intercalator mitoxantrone and the minor groove binding distamycin (Dist), both of which can inhibit the binding of E2F1 to the dihydrofolate reductase promoter, are strong inhibitors of dihydrofolate reductase gene expression (11). However, most DNA binding ligands are not promoter- or TF-specific inhibitors; Dist, for example, is also known to inhibit gene transcription by interfering with the association of TATA box-binding protein to its A,T-rich response element (TATA box) found in the proximal promoter of many genes (12).

We have been investigating a new class of DNA minor groove binding ligands, hairpin pyrrole-imidazole polyamides, as potential promoter- and TF-specific inhibitors of gene expression. In this study we have designed several new polyamides specifically targeted to the Ets binding site (EBS) within the proximal promoter of the HER2/*neu* oncogene. Hairpin polyamides represent a significant advancement in ligand design in that they can achieve a remarkable degree of sequence specificity and high affinity for predetermined DNA sequences (13–15). Polyamides that contain the aromatic rings *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) bind as pairs in an antiparallel fashion to specifically distinguish G·C (Im/Py) from C·G (Py/Im). Py/Py pairs are partially degenerate and bind both A·T and T·A pairs. More than five aromatic rings are overwound relative to the DNA helix, and a β-alanine unit has proven to be a conformationally flexible analog of a pyrrole carboxamide unit (15). A β/β pair can replace a Py/Py pair and allow for recognition of longer sequences while maintaining the specificity for AT/TA base pairs (15). Recently, polyamides designed to interfere with TFIIIA binding to its promoter-response ele-

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** To whom correspondence may be addressed: Div. of Chemistry and Chemical Engineering and Beckman Institute, California Institute of Technology, Pasadena, CA 91125. Tel.: 626-395-6002; Fax: 626-683-8753; E-mail: dervan@cco.caltech.edu.

§§ To whom correspondence may be addressed: Dept. of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Tel.: 716-845-3443; Fax: 716-845-8857; E-mail: terry.beerman@roswellpark.org.

[1] The abbreviations used are: TF, transcription factor; Dist, distamycin; EBS, Ets binding site; Im, *N*-methylimidazole; Py, *N*-methylpyrrole; bp, base pair(s); AP, activator protein; EMSA, electrophoretic mobility shift assay; ESX, epithelial restricted with serine box.

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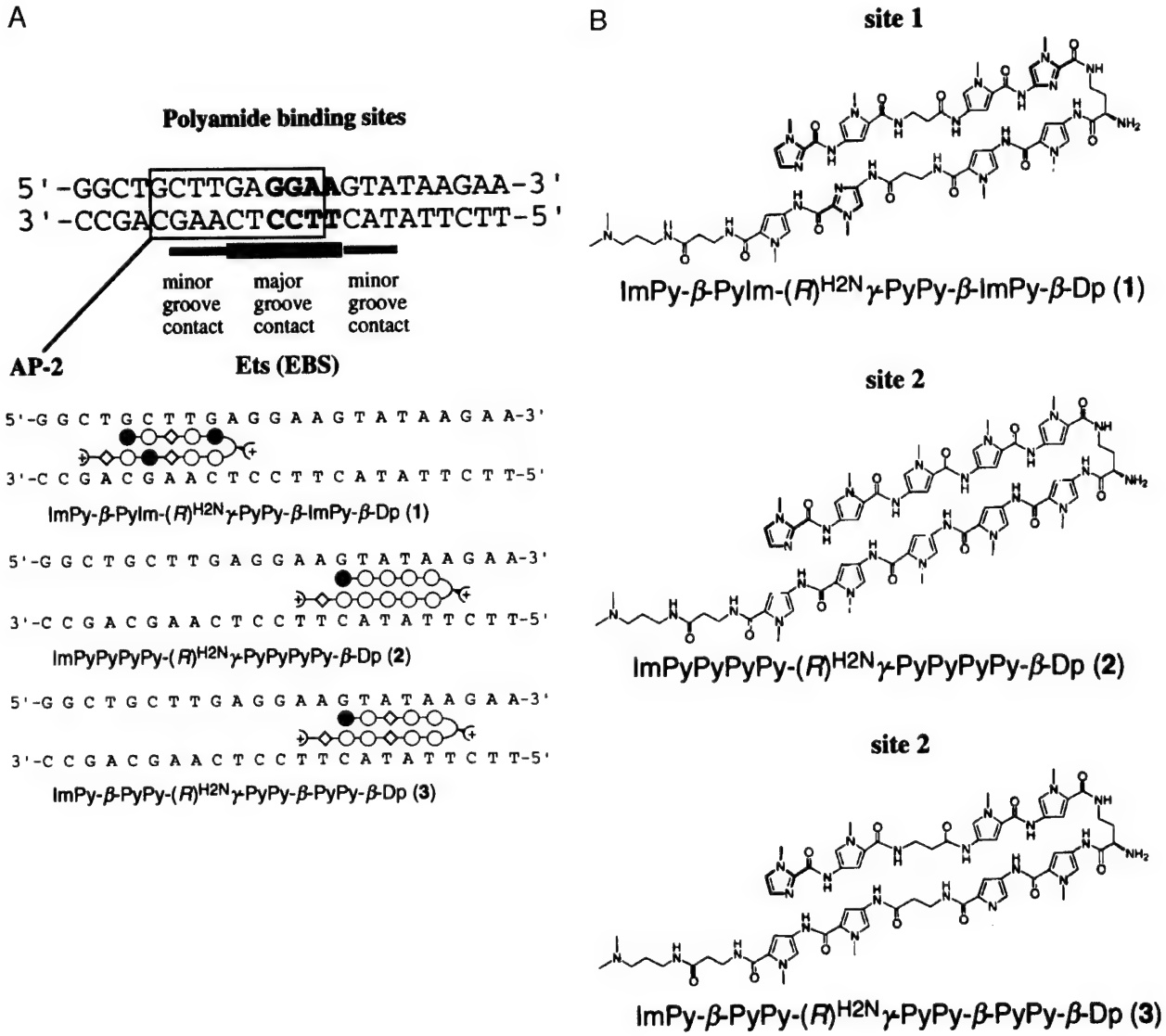

FIG. 1. *A*, HER2/*neu* promoter sequence (and TA5 probe) containing Ets (EBS), AP-2, and TATA box-binding protein-response elements, and showing the 7-bp polyamide binding elements overlapping and positioned just upstream (for polyamide **1**) and downstream (for polyamides **2** and **3**) of the GAGGAA EBS. Schematic binding model of the polyamides; imidazole and pyrrole rings are represented as *shaded* and *unshaded spheres*, respectively, whereas the β-alanine residues are represented as *unshaded diamonds*. *B*, structure of polyamides ImPy-β-PyIm-$(R)^{H2N}$γ-PyPy-β-ImPy-β-Dp (**1**), ImPyPyPyPy-$(R)^{H2N}$γ-PyPyPyPy-β-Dp (**2**), and ImPy-β-PyPy-$(R)^{H2N}$γ-PyPy-β-PyPy-β-Dp (**3**).

ment, were shown to be potent and specific inhibitors of 5 S RNA gene transcription (16, 17). Such designed polyamides have also been shown to specifically inhibit the replication of human immunodeficiency virus, type I virus within the genome of human blood cells (18).

The HER2/*neu* oncogene is amplified and transcriptionally up-regulated in 25–30% of human breast cancers (19). The dramatic loss of ErbB2/HER2 promoter activity in overexpressing (MDA-453) and normal expressing (MCF-7) cells when a mutation of the ErbB2/HER2 promoter's EBS is introduced (GAGGAA to GAGAGA) into a transfected ErbB2 promoter-chloramphenicol acetyltransferase reporter construct demonstrates that the transcriptional up-regulation of HER2/*neu* depends on a highly conserved EBS and its GAGGAA core recognition sequence within the key regulatory region of the HER2/*neu* proximal promoter (20). Recent studies have confirmed, both *in vitro* and *in vivo*, that ErbB2-mediated tumorigenesis could be inhibited by transfecting an Ets repressor that binds specifically and uniquely to the same ErbB2/HER2 promoter's EBS being targeted by our polyamide ligands (21). A number of polyamides were designed to target the EBS and adjacent upstream or downstream flanking sequences unique to this promoter. Three different hairpin polyamides ImPy-β-PyIm-$(R)^{H2N}$γ-PyPy-β-ImPy-β-Dp (**1**), ImPyPyPyPy-$(R)^{H2N}$γ-PyPyPyPy-β-Dp (**2**), and ImPy-β-PyPy-$(R)^{H2N}$γ-PyPy-β-PyPy-β-Dp (**3**) were synthesized to target either the upstream or downstream EBS flanking sequences 5′-TGCTTGA-3′ or 5′-AGTATAA-3′, respectively (Fig. 1*A*). Quantitative footprint titration analysis confirmed their high affinity binding and sequence specificity for the HER2/*neu* EBS. Comparisons were made between these polyamides and the classical three ring DNA minor groove binder, Dist, in their abilities to inhibit binding to the HER2/*neu* EBS by the mammary gland Ets factor, ESX, thought to endogenously regulate this promoter in HER2/*neu* overexpressing human breast cancers (26). Lastly, a cell-free transcription assay was used to evaluate the specific and differential ability of these three polyamides to interfere with HER2/*neu* promoter-driven transcription.

MATERIALS AND METHODS

Synthesis of the Polyamides—The polyamides ImPy-β-PyIm-$(R)^{H2N}$γ-PyPy-β-ImPy-β-Dp (**1**), ImPyPyPyPy-$(R)^{H2N}$γ-PyPyPyPy-β-Dp (**2**), and

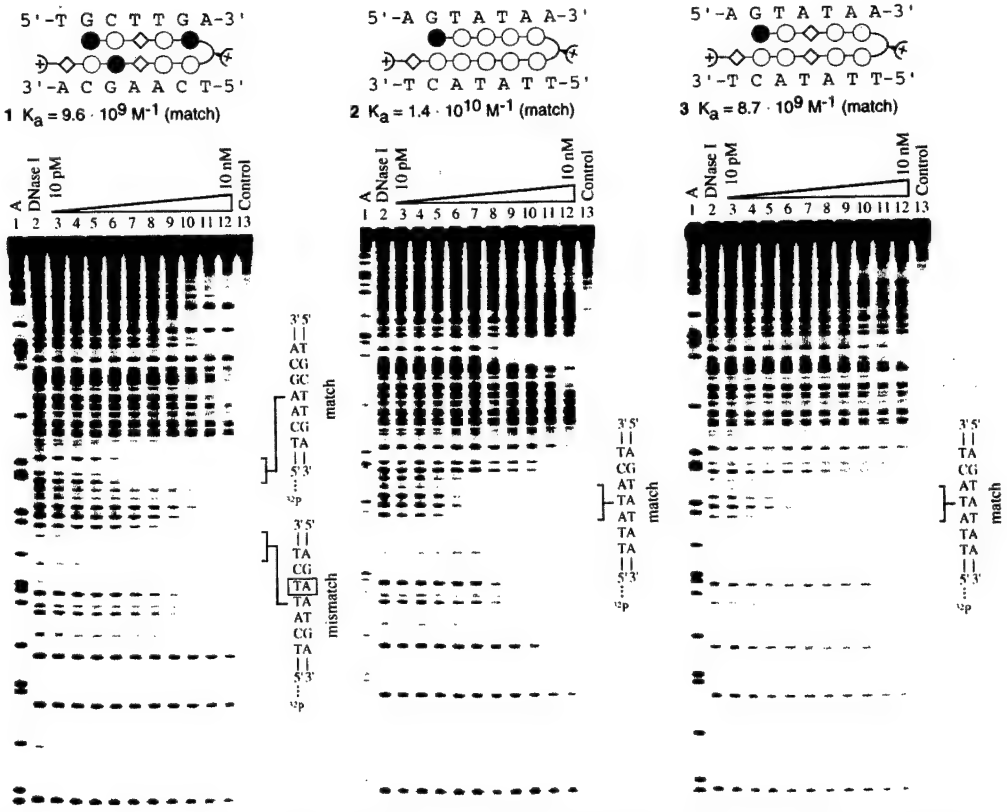

FIG. 2. **Quantitative DNase I footprint titration experiment with the polyamides 1, 2, and 3 on a 5′-^{32}P-radiolabeled, 188-base pair DNA fragment obtained by polymerase chain reaction from the plasmid RO6.** *Lane 1*, A reaction; *lane 2*, DNase I standard; *lanes 3–12*, 10 pM, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 5 nM, and 10 nM polyamide; *lane 13*, intact DNA. All reactions contain a 17-kcpm DNA fragment, 10 mM Tris·HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

ImPy-β-PyPy-(*R*)^{H2N}γ-PyPy-β-PyPy-β-Dp (**3**) were synthesized from β-alanine-PAM resin using solid phase as described (22) and was characterized by a combination of analytical high pressure liquid chromatography, UV spectroscopy, and matrix-assisted laser desorption ionization/time of flight mass spectroscopy. MS, $^m/_z$ observed for **1,** 1380.7; 1380.7 calculated for $[M + H]^+$; $^m/_z$ observed for **2,** 1480.6, 1480.7 calculated for $[M + H]^+$; $^m/_z$ observed for **3,** 1378.6, 1378.7 calculated for $[M + H]^+$. UV in $M^{-1}\ cm^{-1}$, for **1,** 42,500 (ϵ_{242}), 53′100 (ϵ_{298}); for **2,** 58,300 (ϵ_{246}), 79′600 (ϵ_{316}); for **3,** 50,000 (ϵ_{240}), 53′600 (ϵ_{298}).

Quantitative DNase I Footprint Titrations—A 188-base pair (bp) DNA fragment was obtained by polymerase chain reaction using the plasmid RO6 as a template and the primers P1 (5′-GAGAAAGT-GAAGCTGGGAGTT-3′) and P2 (5′-CCTGGTTTCTCCGGTCCCAAT-3′). The primer P2 was 5′-radiolabeled with [γ-^{32}P]ATP using T4-polynucleotide kinase (Roche Molecular Biochemicals). Polymerase chain reaction amplification in the presence of P1, P2 (labeled), plasmid RO6, and *Taq* polymerase (Roche Molecular Biochemicals) gave a 188-bp DNA fragment that was purified on a 6% nondenaturating polyacrylamide gel. All DNase I footprint reactions were performed in a total volume of 400 μl containing a 5′-^{32}P-radiolabeled DNA fragment (17,000 cpm) and final concentrations of 10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.0, and either 0.1–10 nM polyamide or no polyamide (control lanes) (23). The solutions were allowed to equilibrate for 12–14 h at 22 °C. The footprinting reactions were initiated by the addition of 10 μl of a stock solution of DNase I containing 1 mM dithiothreitol and incubated for 7 min at 22 °C. The reactions were stopped by adding 50 μl of a solution of 2.25 M NaCl, 150 mM EDTA, 28 mM base pair calf thymus DNA, and 0.6 mg/ml glycogen and ethanol precipitation. The precipitates were resuspended in 1× Tris borate-EDTA, 80% formamide loading buffer, denaturated by heating at 85 °C for 10 min, and cooled on ice. The reaction products were separated by electrophoresis on a 6% polyacrylamide gel in 1× Tris borate-EDTA at 2000 V for 1 h. Gels were dried on a slab dryer and exposed to a photostimulatable storage phosphorimaging plate (Kodak Storage Phosphor Screen SO230 obtained from Molecular Dynamics) in the dark at 22 °C for 12–24 h. The data from the storage screens were obtained using a Molecular Dynamics 400S PhosphorImager and analyzed by volume integration of the target sites and reference blocks using the ImageQuant version 3.3. software. Equilibrium association constants were determined as described previously (24). Each compound was tested three times; the values for the equilibrium association constants (K_a) correspond to average values from three independent gels.

Cell Culture and Nuclear Extract Preparation—SKBR-3 cells were purchased from ATCC (Rockville, MD) and maintained at 37 °C with 5% CO_2 and in McCoy's 5a medium (Life Technologies, Inc.) with 10% fetal bovine serum. SKBR-3 cells grown to subconfluence were rinsed twice with phosphate-buffered saline, scraped, and collected by centrifugation at 1200 rpm for 5 min, 4 °C (Sorvall RT6000, Newtown, CT). The following steps were performed at 4 °C. Cell pellets were suspended in five times the packed cell volume in buffer A (containing 10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, and 1 mM dithiothreitol), followed by centrifugation at 1200 rpm for 5 min. The pellet was then resuspended in five times the pellet volume in buffer A, kept on ice for 8 min, and homogenized with 10 strokes using a Dounce homogenizer (tight pestle). The homogenate (~95% lysed cells) was centrifuged at 15,000 rpm for ~1 min, (JA-17 rotor, JA-21 centrifuge; Beckman, Palo Alto, CA). The pellet was resuspended in buffer B with 20 mM Hepes-KOH (pH 7.9), 20% glycerol, 0.2 mM EDTA, 2.0 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride followed by drop addition of an equal volume of buffer B that included 0.75 M NaCl. After rocking for 20 min, the supernatant was collected by centrifugation at 47,500 rpm for 45 min (SW-55 rotor, Beckman), and dialyzed against >100-fold buffer C (20 mM Hepes-KOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) for 3 h. Precipitated debris was removed by centrifugation at 15,000 rpm (JA-21 centrifuge, JA-17 rotor, Beckman), and the protein content of the nuclear extract was quantitated using the Bio-Rad protein assay.

Proteins, Antibodies, and Oligonucleotides—Recombinant ESX protein was prepared as described (25). Briefly, full-length ESX cDNA was cloned into a pRSET His tag expression plasmid (*Nhe*I-*Hin*dIII; Invitrogen) and expressed in isopropyl-1-thio-β-D-galactopyranoside-induced BL21[DE3] pLysS competent bacteria (Stratagene, La Jolla, CA). His-

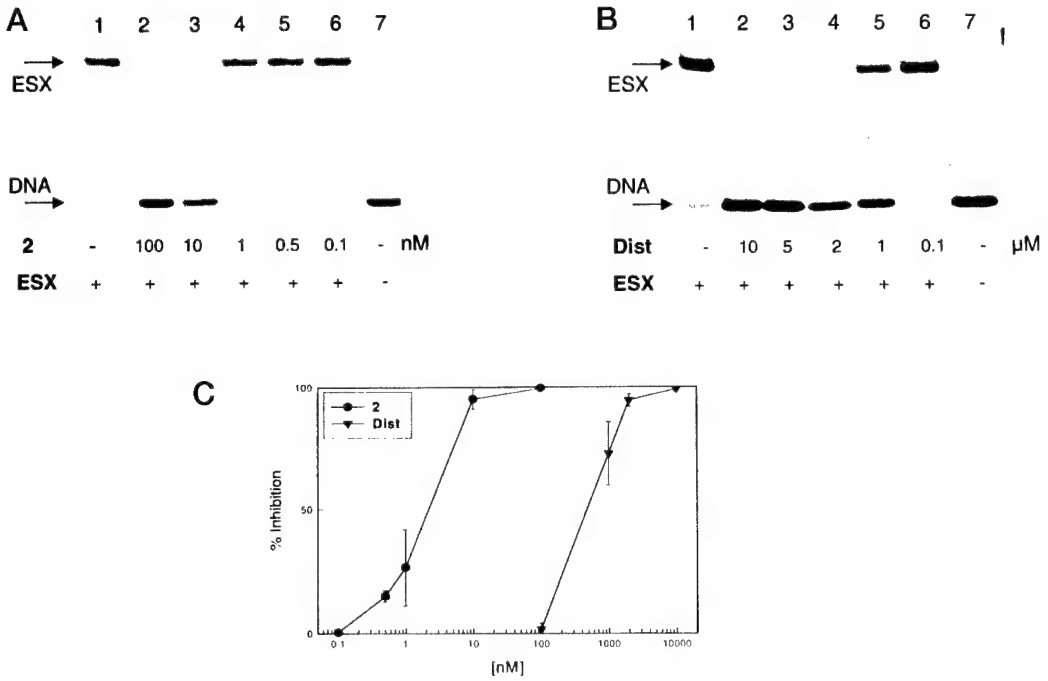

FIG. 3. **EMSA comparison of polyamide 2 *versus* Dist inhibition of ESX-TA5 complex formation.** *A*, EMSA performed in the presence of **2** was used to evaluate the ability of polyamides to inhibit ESX binding to the labeled TA5 HER2/*neu* promoter probe. As described under "Materials and Methods," labeled TA5 probe and compound were incubated for 30 min at room temperature followed by the addition of ESX and subsequent a 30-min incubation. Complexes formed in solution were then separated on a 5% native polyacrylamide gel and visualized by autoradiography. *Lane 1*, control of ESX-TA5; *lanes 2–6*, samples in the presence of **2** at concentrations of 100, 10, 1, 0.5, and 0.1 nM, respectively; *lane 7*, control of free TA5 probe. *B*, EMSA performed in the presence of Dist under the same assay conditions as described for **2**. *Lane 1*, control of ESX-TA5; *lanes 2–6*, samples in the presence of Dist at indicated concentration of 10, 5, 2, 1, and 0.1 μM, respectively; *lane 7*, control of free probe. *C*, inhibition of ESX-TA5 complex formation in the presence of **2** or Dist, plotted as the percentage of control ESX-TA5 complex formation. **2** (●) and Dist (▼) at the indicated concentrations were incubated with the TA5 probe prior to the addition of recombinant ESX protein. The densitometry quantitated data represent the mean values (± S.D.) from at least three separate experiments.

TABLE I
Effects on TF-DNA complex formation by polyamides

Polyamide	TF	IC_{50}	IC_{50} *r* value[a]
		nM	
1	ESX	5	0.16
	AP-2	48	1.55
2	ESX	2.2	0.07
	AP-2	>100	nd[b]
3	ESX	18	0.58
	AP-2	nd	nd
Dist	ESX	500	16.1
	AP-2	6000	193.5

[a] *r* value, the molar ratio of compound to DNA base pairs.
[b] nd, not determined.

tagged ESX protein was purified by Ni^{2+}-chelate affinity chromatography, as recommended by the manufacturer (Qiagen Inc., Valencia, CA). Recombinant AP-2 protein was purchased from Promega Co. (Madison, WI). Monoclonal antibody against AP-2 was purchased from Santa Cruz Biochemical (Carpenteria, CA), and the anti-ESX affinity-purified rabbit polyclonal was prepared as described previously (26). A 34-mer DNA oligonucleotide (oligo) containing the EBS and derived from the HER2/*neu* proximal promoter (TA5 sequence shown in Fig. 1*A*) and its complementary strand, were synthesized by the Biopolymers facility (Roswell Park Cancer Institute, Buffalo, NY). Oligos were gel-purified, annealed, and 5′-end-labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase (New England BioLabs, Beverly, MA) as described previously (27).

Mobility Shift Assay—Demonstration of TFs binding to their DNA response elements in the proximal HER2/*neu* promoter was performed by EMSA using recombinant TFs (ESX, AP-2), duplexed and 5′-end-labeled TA5 promoter probe, with/without anti-TF antibody. In general, recombinant protein at the indicated concentrations and 1 nM ^{32}P-labeled DNA probe were incubated in a reaction buffer containing 25 mM Tris (pH 7.5), 30 mM KCl, 5% glycerol, 0.1% Nonidet P-40, bovine serum albumin (100 μg/ml), and 1 mM dithiothreitol. After incubation at room temperature for 30 min, samples were loaded onto 5% native polyacrylamide gels running with Tris borate-EDTA buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). The dried gel was exposed to Kodak film and the protein-DNA complexes were quantitated by computing laser densitometry (Molecular Dynamics, Sunnyvale, CA).

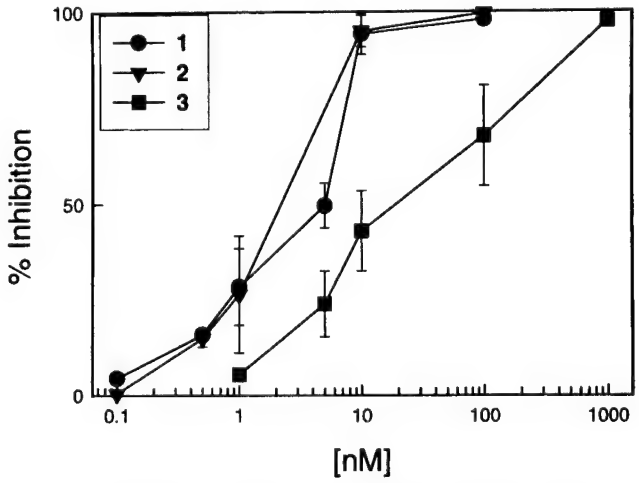

FIG. 4. **Inhibition of ESX-TA5 complex formation after pretreatment of HER2/*neu* promoter probe with various polyamides.** EMSA experiments were performed as described in Fig. 3*A*. Radiolabeled TA5 probe and polyamides **1** (●), **2** (▼), or **3** (■) were incubated for 30 min at room temperature before the addition of recombinant ESX protein. Following gel separation, autoradiography, and densitometry, data are represented as mean values (± S.D.) from three separate experiments.

Identification of specific protein-DNA complexes was confirmed by the addition of specific antibodies in the EMSA reaction conditions, as indicated. The ability of polyamides to interfere with the formation of a protein-DNA complex was determined by EMSA. For polyamide effects

on monomeric ESX binding to TA5 (ESX-TA5 complex formation), assays were performed in two ways: (i) polyamides were incubated with a ^{32}P-labeled probe at room temperature for 30 min prior to the addition of ESX protein, and (ii) ESX protein was complexed with the probe prior to polyamide treatment. The inhibition of ESX-TA5 complex formation was measured by comparing polyamide-treated with nontreated samples. Polyamide ability to inhibit dimeric AP-2 binding to TA5 (AP-2-TA5 complex formation) was measured in a similar manner. IC_{50} values (concentration of compound required for 50% inhibition of protein-DNA complex formation) were determined to express the inhibitory activity of each agent; these IC_{50} polyamide concentrations were also expressed as *r* values, the molar ratio of ligand to DNA base pairs.

Cell-free Transcription Assay—In vitro transcription was performed in a cell-free system composed of DNA template, SKBR-3 nuclear extract, and buffer containing 12 mM Hepes-KOH (pH 7.9), 60 mM KCl, 7.5 mM $MgCl_2$, 12% glycerol, 0.12 mM EDTA, 0.12 mM EGTA, 1.2 mM dithiothreitol, and 0.6 mM phenylmethylsulfonyl fluoride. The transcription DNA template consisted of CsCl-purified plasmid DNA containing the ~500-bp RO6 HER2/*neu* promoter fragment (20), inserted into a pCDNA3-Luc expression vector (Invitrogen, Carlsbad, CA), and linearized by restriction with *Sph*I (New England BioLabs, Beverly, MA). Into a 25-μl reaction of SKBR-3 nuclear extract was added 1 μg of *Sph*I-digested DNA, nuclear extracts, 0.5 μl of each nucleotide (20 mM of ATP, GTP, UTP, and 100 μM CTP), 10 μCi of [α-^{32}P]CTP (800 Ci/mmole; NEN Life Science Products), 1 μl of RNAsin (40 units/μl; Roche Molecular Biochemicals), and 1.4 μl of EDTA (2.5 mM). Transcript formation proceeded with incubation at 30 °C for 60 min, and the reaction was stopped by adding 325 μl of 10 mM Tris base (pH 8.0), 7 M urea, 350 mM NaCl, 1% SDS, and 100 μg of tRNA, followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. Samples were resuspended in formamide-loading dye and heated at 90–95 °C for ≥1 min before loading onto a 4%, 7 M urea-polyacrylamide gel. The ^{32}P signal from a dried gel was visualized using a PhosphorImager screen and quantitated by computing laser densitometry (Molecular Dynamics, Sunnyvale, CA).

As with EMSA assessment of polyamide activity, ligand ability to inhibit transcript formation driven off the HER2/*neu* promoter was analyzed in two ways: (i) DNA template was incubated with polyamide at the indicated concentration in a total volume of 10 μl for 30 min prior to the addition of nuclear extract and radiolabeled nucleotide pool, and (ii) preincubation of nuclear extract and DNA template for 15 min was followed by the addition of ligand for another 30 min in the total reaction volume to which radiolabeled nucleotide pool was then added. The degree of transcription was measured by quantitating transcript formation in ligand-treated *versus* untreated (control) samples and calculating IC_{50} and *r* values. T3 transcript (250 bases; Promega Co., Madison, WI) was used as an internal control. In addition, a time course assay was used to compare transcriptional inhibition off the HER2/*neu* promoter in the presence of Dist *versus* polyamide **2** using our previously described procedure (11). For these time course assays, following the addition of ligands and nucleotides to the premixed template and nuclear extract volume, the reaction was stopped at different time points (0–60 min), and the newly formed transcripts were quantitated as described above.

RESULTS

Design of HER2/neu Promoter Binding Polyamides—We used the simple pairing code (15) to design polyamides that bind the 5′- and 3′-flanking sequences overlapping the EBS (GAGGAA) within the endogenous HER2/*neu* proximal promoter, the RO6 HER2/*neu* promoter-driven transcript template, and the EMSA TA5 probe. The proximal HER2/*neu* promoter sequence containing this EBS is shown in Fig. 1*A*, and this HER2/*neu* EBS has previously been shown to bind with high affinity to the potent and epithelial-restricted Ets transactivator, ESX (25, 26). All Ets family members bind to the major groove of DNA and have additional critical phosphate contacts along flanking minor groove sequences (28, 29). Whereas the GGAA core of the EBS is the same for virtually all Ets proteins, these 5′- and 3′-sequences immediately adjacent to the EBS core are often promoter- and Ets factor-specific. Thus, we synthesized three polyamides to target 7 bp of the HER2/*neu* promoter sequence 5′ and 3′ of the EBS core; polyamide **1** was designed to bind immediately upstream of the ESX core binding site at 5′-TGCTTGA-3′ (site 1), whereas

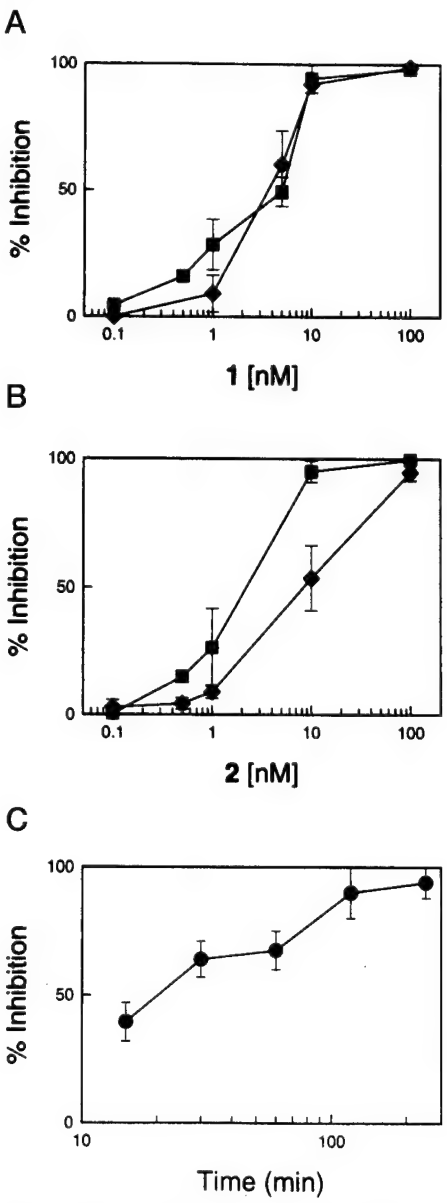

FIG. 5. **Comparative HER2/*neu* inhibitory effects of polyamides administered before or after formation of ESX-TA5 complexes.** EMSA performed when **1** (*A*) or **2** (*B*) were added at indicated concentrations either before (■) or after (♦) formation of ESX-TA5 complexes. Following gel separation, autoradiography, and densitometry, data are represented as mean values (± S.D.) from at least three separate experiments. A time course assay was used to estimate the time required for **2** to reach steady-state equilibrium in terms of inhibition of ESX-TA5 complex formation (*C*). ESX and radiolabeled TA5 probe were incubated at room temperature for 30 min followed by the addition of polyamide **2** at 10 mM for 240, 120, 60, 30, or 15 min; replicate assay results are expressed as mean values (± S.D.).

polyamides **2** and **3** were designed to bind immediately downstream and across the adjacent TATA box at 5′-AGTATAA-3′ (site 2).

Selectivity and High Affinity Binding of Polyamides to the HER2/neu Promoter EBS—Quantitative DNase I footprint titration analysis showed that polyamides **1, 2,** and **3** bind with high affinity to their target sites (Fig. 2). Polyamide **1** binds with an equilibrium association constant $K_a = 9.6 \cdot 10^9\ M^{-1}$ to its match site (5′-TGCTTGA-3′). It binds by a factor of ~2 less strongly to a single base pair mismatch site (5′-AGAATGA-3′) located downstream with respect to the ESX binding site ($K_a = 4.5 \cdot 10^9\ M^{-1}$). Polyamides **2** and **3** both bind with high affinity

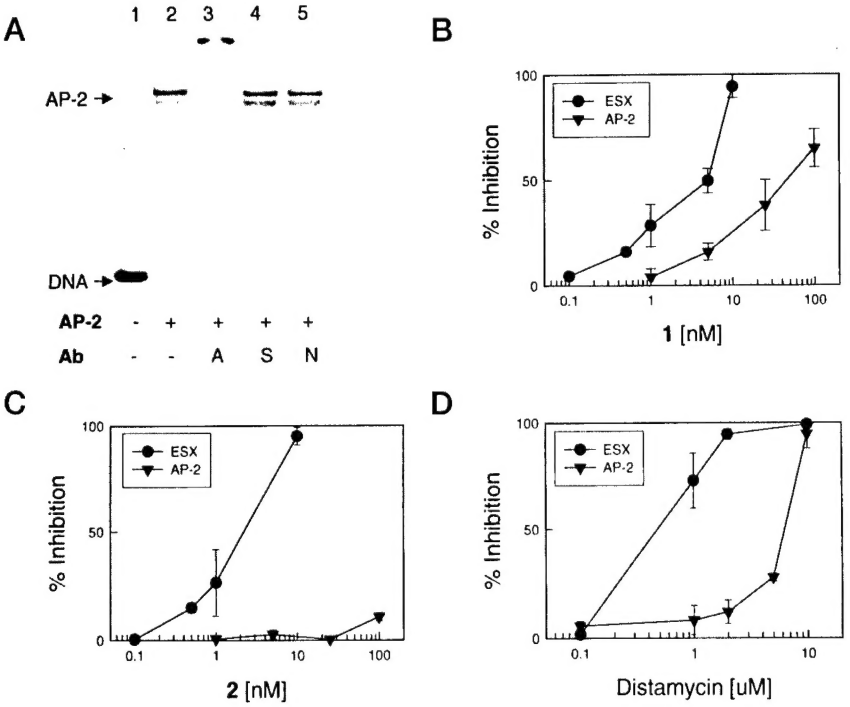

FIG. 6. **Antibody-specific AP-2 binding to the HER2/*neu* promoter probe TA5, and polyamide *versus* Dist inhibition of AP-2-TA5 complex formation.** Demonstration of specific AP-2 binding to TA5 confirmed by AP-2 antibody-induced supershifting on EMSA. Recombinant AP-2 protein with/without monoclonal antibodies were incubated for 10 min prior to the addition of radiolabeled TA5 probe, as described under "Materials and Methods." *A, lane 1*, control of free TA5 probe; *lane 2*, AP-2-TA5 complexes; *lanes 3–5*, reactions with antibodies of *A* (AP-2), *S* (Sp1), and *N* (normal immunoglobulins), showing formation of a slower migrating (supershifted) TA5 complex only in the presence of AP-2-TA5 binding monoclonal. Inhibition of the AP-2-TA5 complexes (▼) was compared with EMSA formation of ESX-TA5 complexes (●) in the presence of (*B*) **1**, (*C*) **2**, or (*D*) Dist. All results are expressed as mean values (± S.D.) from replicate experiments each performed with duplicate samples.

to their target site (5′-AGTATAA-3′, $K_a = 1.4 \cdot 10^{10}$ M^{-1} and $K_a = 8.7 \cdot 10^9$ M^{-1}, respectively).

Polyamide 2 Inhibition of Ets Binding to the HER2/neu Promoter—Because Dist can also bind to the TATA box contained in the 3′-EBS element targeted by two of the polyamides (12), polyamide **2** and Dist were compared by EMSA for their abilities to inhibit ESX binding to the HER2/*neu* promoter probe, TA5. Incubation of **2** with TA5 followed by the addition of ESX resulted in a concentration-dependent inhibition of ESX-TA5 complex formation; 10 nM **2** inhibited complex formation up to 95%, whereas as little as 1 nM resulted in a detectable decrease in complex formation (Fig. 3*A*, *lanes 2–4*). The pattern of inhibition of ESX-TA5 complex formation by Dist was similar, but significantly higher Dist concentration was required to achieve the same degree of inhibition observed by **2**, because Dist at 2000 nM diminished complex formation by ~95% (Fig. 3*B*, *lane 4*). Whereas 100 nM of **2** inhibited ESX-TA5 complex formation almost entirely, 100 nM Dist had no effect on ESX-TA5 complex formation (Fig. 3*A*, *lane 2* and Fig. 3*B*, *lane 6*). Quantitation of the data in Fig. 3*C* indicated that 2.2 nM **2** and 500 nM Dist are needed to inhibit complex formation by 50% (IC_{50}); Table I also shows the activity of individual polyamides at inhibiting protein-DNA complex formation expressed as *r* values, the molar ratio of ligand to DNA base pairs.

Inhibitory Effects of Polyamides (1, 2, and 3) on Ets Binding to the HER2/neu Promoter—Because **1** and **2** recognize DNA elements upstream and downstream of the core EBS while **3** also recognizes the same downstream flanking element as **2** (Fig. 1), EMSA was used to test the relative ability of each polyamide to inhibit ESX binding to the HER2/*neu* promoter probe TA5. As shown in Fig. 4, **1** and **2** appeared similar in their ability to inhibit complex formation with respective IC_{50} values of 5 and 2.2 nM. In contrast, **3** required a 9-fold higher concentration (18 nM) to prevent Ets-DNA complex formation by 50% as compared with **2** (Fig. 4 and Table I).

For certain TF/DNA inhibitory drugs, equilibrium conditions demand greater drug concentrations to inhibit preformed DNA-bound complexes as opposed to preventing the initial formation of such complexes (6). For polyamide **1**, however, similar experimental conditions were observed for inhibition of Ets-DNA complexes whether ligand was added before or after ESX binding to the HER2/*neu* promoter probe, because at 10 nM polyamide **1** concentration nearly the same level of ESX-TA5 complexes were formed within 30 min no matter which order the reagents were added (Fig. 5*A*). In contrast, **2** required 10-fold more ligand to obtain equal inhibition when added after ESX-TA5 complex formation as compared with the addition of **2** before complex formation (Fig. 5*B*). A time course assay using 10 nM of **2** indicated that the percentage of ESX-TA5 complexes inhibited by **2** increased with longer incubation time such that a ≥4 h polyamide **2** incubation was needed to achieve equilibrium conditions and maximal inhibition when **2** was added after the initial formation of ESX-TA5 complexes (Fig. 5*C*).

Inhibitory Effects of Polyamides on AP-2 Versus Ets Binding to the HER2/neu Promoter—Previous studies have suggested that AP-2 contributes to the overexpression of HER2/*neu*, and footprinting analysis has revealed that there are several AP-2 sites in the proximal HER2/*neu* promoter (30). We also had observed that both endogenous and recombinant AP-2 binds to a G-rich sequence just upstream of the EBS on the HER2/*neu* promoter. Dimeric binding of AP-2 to this G-rich element in the TA5 probe (Fig. 1) is demonstrable by EMSA and confirmed by the supershifting effect of an AP-2 monoclonal; in contrast, antibodies nonreactive to AP-2 had no effect on this AP-2-TA5 complex (Fig. 6*A*). Because AP-2 interacts with this G-rich element adjacent to the EBS in TA5, it was of interest to know whether the EBS-targeted polyamides would affect the binding of AP-2 to this HER2/*neu* promoter probe. Fig. 6*B* shows that **1**

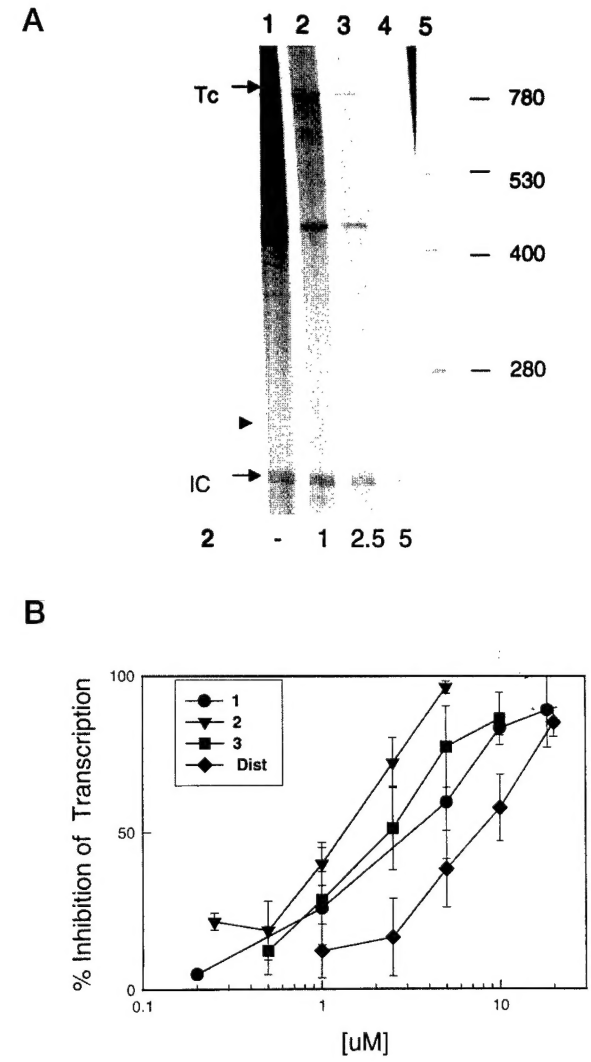

FIG. 7. **Polyamide and Dist pretreatment of the HER2/*neu* promoter inhibits its transcriptional activity.** HER2/*neu* promoter-driven transcription was measured in a cell-free assay as described under "Materials and Methods"; briefly, compound at the indicated concentration was incubated with a *Sph*I-restricted HER2/*neu* promoter-driven transcription template (RO6) at 30 °C for 30 min, followed by the addition of SKBR-3 nuclear extract and a radiolabeled pool of nucleotide precursors. Transcription was allowed to proceed for 60 min at 30 °C with the expected ~760-base transcript identified by gel electrophoretic separation and phosphorimaging of the scanned gel. Scan from a representative experiment performed in the presence of polyamide **2** is shown in *A*. *Lane 1*, untreated control; *lanes 2–4*, cell-free transcription performed in the presence of 1, 2.5, and 5 μM **2**; *lane 5*, RNA marker. *IC*, internal control; *TC*, 760-base transcript. Activities of polyamides, **1** (●), **2** (▼), **3** (■), and Dist (♦) are presented as percentage inhibition of transcript formation comparing the compound-treated condition with untreated control (*B*) and transcript formation in individual samples normalized to the internal control. Results represent the mean values (± S.D.) of replicate experiments.

was capable of inhibiting EMSA-detected AP-2-TA5 complexes in a concentration-dependent manner and with an IC_{50} = 48 nM. In contrast, **2** was unable to block complex formation even at the highest concentration (100 nM) tested (Fig. 6*C*).[2] Likewise, the pattern of inhibition of AP-2-TA5 complexes by Dist was similar to that of **2** in that micromolar concentrations were required to significantly inhibit complex formation (Fig. 6*D* and Table I). All these compounds were more efficient at inhibiting formation of ESX (*versus* AP-2) complexes on the HER2/*neu* promoter probe, with **2** being the most specific and most potent inhibitor.

[2] Polyamide concentration greater than 100 nM caused smearing of the DNA under our assay conditions.

TABLE II
Effects on the in vitro transcription by polyamides

Polyamide	IC_{50}	IC_{50} *r* value
	μM	
1	3.2	0.02
2	1.4	0.009
3	2.4	0.015
Dist	7.4	0.05

Transcription Inhibiting Effects of Polyamides on the HER2/neu Promoter—To determine whether the effects of polyamides on Ets-DNA complex formation resulted in an ability to influence biological function, *in vitro* transcription assays were performed using a HER2/*neu* promoter-driven DNA template. With the ~500 bp of HER2/*neu* promoter-inserted plasmid (RO6) linearized with *Sph*I as DNA template and SKBR-3 nuclear extracts (endogenously enriched in ESX, AP-2, TATA box-binding protein, etc.) to provide the transcriptional machinery, a ~760-base transcript is produced in this cell-free system. Compounds were first incubated with the DNA template prior to the addition of nuclear extracts and radiolabeled pool of nucleotides. A representative gel shown in Fig. 7*A* demonstrates the ability of **2** to block synthesis of the 760-base transcript in a concentration-dependent manner. Compared with the untreated control, 5 μM of **2** inhibited transcript synthesis by 95%; whereas 1 μM produced less than 50% inhibition of transcript formation, at higher polyamide concentrations there was some evidence of the partial transcript production (Fig. 7*A*, *lanes 3* and *4*). Comparative inhibition of HER2/*neu* promoter-driven transcription by **1, 2, 3,** and Dist is shown in Fig. 7*B*. The order of transcription inhibiting potency (**2** > **3** > **1** > Dist) is somewhat different from the EMSA ESX-TA5 complex inhibiting potency for these compounds. Their corresponding IC_{50} values are 1.4 μM for **2,** 2.4 μM for **3,** 3.2 μM for **1,** and 7.4 μM for Dist; their *r* values are also shown in Table II.

Because EMSA results demonstrated differences between the ability of **1** and **2** to inhibit Ets-DNA complexes when ligand was given before or after initial formation of the ESX-TA5 complex (Fig. 5), to determine if the order of addition of polyamides influenced their transcription inhibitory activity, nuclear extracts were allowed to interact with the promoter and DNA template prior to ligand exposure. Polyamide effectiveness appeared to be reduced when tested in this fashion (Fig. 8). For example, concentrations of **2** at 1.4 or 4.2 μM were required to inhibit transcription by 50% when ligand was added before or after nuclear extract binding with the R06 template (Fig. 8*A*). In the case of **1,** a 2-fold higher ligand concentration was needed to inhibit transcription (6.4 μM) when extract was prebound to template (Fig. 8*B*).

Previous time course studies with DNA binding and transcription-inhibiting drugs have shown that the degree of transcription inhibition can change in relation to the *in vitro* reaction time in the presence of moderately inhibiting drug concentrations (11). Conducting similar time course experiments with the most potent inhibitor **2** revealed a plateau level of transcription inhibition at all time points from 10–60 min (Fig. 9). In contrast, the level of transcription inhibition by Dist declined somewhat in relation to incubation time. These time course differences between **2** and Dist might be accounted for by the higher DNA binding affinity of the polyamide, making it less likely that ligand is released from the template and transcription is allowed to resume during the longer exposure times.

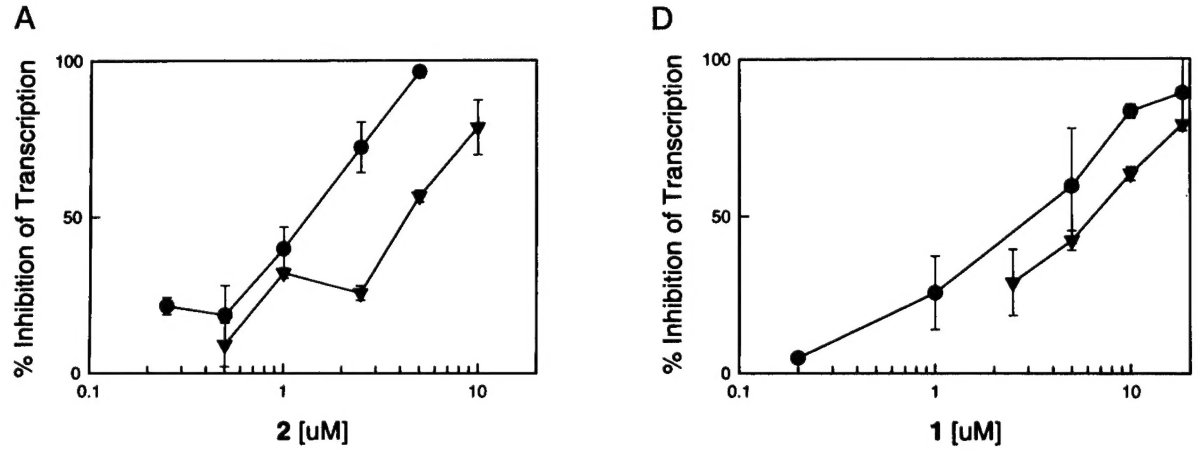

FIG. 8. **Comparative inhibition of HER2/*neu* promoter-driven transcription when polyamides are administered before or after promoter binding to endogenous transcription factors contained in SKBR-3 nuclear extract.** The experimental procedure was similar to that described in Fig. 7*A* except that the promoter-driven DNA template was incubated with SKBR-3 nuclear extracts for 15 min before the addition of a labeled pool of nucleotide precursors and polyamides **2** (*A*) or **1** (*B*). The percent inhibition of transcript formation (▼) produced by the individual ligands was compared with that produced when ligand exposure to template preceded the addition of nuclear extract (●). Results represent the mean (± S.D.) of replicate experiments.

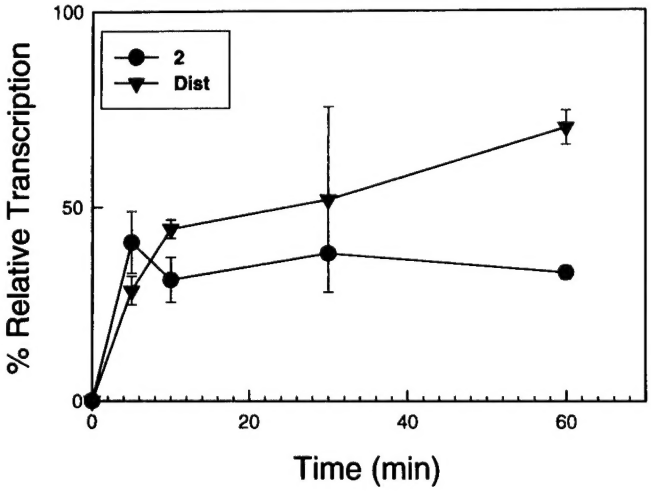

FIG. 9. **Inhibition of HER2/*neu* promoter-driven transcription when compound is administered after template binding to nuclear extract and as a function of exposure time.** The experimental procedure was similar to that described in Fig. 8 except that the cell-free transcription reaction was stopped after a 5-, 10-, 30-, or 60-min exposure to **2** (●) and Dist (▼). Results represent the mean (± S.D.) of replicate experiments.

DISCUSSION

In this study, we examined the ability of sequence-specific polyamides to inhibit Ets-DNA complex formation and EBS-regulated transcription off the HER2/*neu* promoter. Polyamides were synthesized that recognize different elements overlapping and flanking the GAGGAA EBS, located adjacent to and 5′ of the TATA-box in the regulatory portion of the proximal HER2/*neu* promoter (20). As compared with the TATA box binding natural product Dist, three designed sequence-specific polyamides were more effective at inhibiting EBS complex formation with the mammary gland Ets transactivator, ESX, as well as HER2/*neu* driven transcription from a ~500 bp HER2/*neu* promoter sequence known to be regulated at the EBS as well as other endogenous response elements (*e.g.* AP-2, Sp1, CAAT, and TATA boxes). Of the three polyamides, **2** was the most strongly binding and effective HER2/*neu* promoter inhibitor, binding with a $K_a = 1.4 \cdot 10^{10}$ M^{-1} to the 3′-flanking EBS element that includes the promoter's TATA box.

Comparison of the three polyamides (**1-3**) with Dist for inhibition of protein-DNA complex formation on the HER2/*neu* promoter probe, TA5, revealed the vastly enhanced potency and specificity of the high affinity hairpins as opposed to the latter natural product. Because both Dist and polyamides **2** and **3** bind the same TATA box containing the 3′-EBS element (Fig. 1), the higher binding affinity of **2** for this element likely contributed to its greater inhibitory activity over both Dist and polyamide **3**. However, because Ets family members also make minor groove phosphate contacts in addition to their major groove base contacts, some of the enhanced inhibitory effects of both these polyamides over Dist may be attributed to steric effects restricting Ets (ESX) access to the HER2/*neu* EBS (28, 31). With a similar comparison in the present study, HER2/*neu* promoter-targeted polyamides were shown to differentially affect ESX and AP-2 binding to adjacent DNA response elements. The binding of polyamide **1** to its 5′-EBS element partially impinges on the G-rich AP-2 binding site present in the TA5 probe (Fig. 1), probably accounting for the observed ~10-fold less effective inhibitory activity of **1** at blocking formation of AP-2-TA5 *versus* ESX-TA5 complexes (Fig. 6*B*). Comparing Dist and polyamide **2** (Fig. 6, *C* and *D*), both of which bind the same 3′-EBS element located more remote from the AP-2 binding element in TA5, demonstrated the vastly improved promoter specificity of a designed polyamide over a less specific natural product like Dist, because the latter showed some AP-2 inhibitory activity whereas the former showed none despite its potent ESX-TA5 inhibitory activity over the same concentration range.

Small molecules that bind DNA near or at a TF response element typically require more time (or higher concentrations) to achieve steady-state inhibition of protein-DNA complexes when added after rather than before the formation of these complexes (6, 12, 27). Differences in this regard were noted between polyamides **1** and **2** when EMSA was carried out with ligands added before or 30 min after formation of ESX-TA5 complexes; polyamide **1** showed no significant impact by delayed administration but **2** showed a near 50% increase in its IC_{50} (Fig. 5, *A* and *B*). However, by increasing its post-treatment incubation time from 30 min to 240 min, a 10 nM dose of polyamide **2** regained its full inhibitory activity as seen with a 30-min pretreatment at this same dose (Fig. 5*C*), demonstrating that **2** required longer post-treatment exposure than **1** to achieve its steady-state inhibitory potential. The difference in

this regard between polyamides **1** and **2** likely reflects 3′-*versus* 5′-asymmetry in the TA5-bound ESX complex, resulting in greater structural interference and reduced access to the TA5 element recognized by **2** in the presence of preformed ESX-TA5 complexes (Fig. 9).

Polyamide **2,** which most effectively inhibited ESX-TA5 complex formation at equilibrium also most effectively inhibited HER2/*neu* promoter-driven transcription, assayed in a cell-free system utilizing endogenous ESX, AP-2, TATA box-binding protein, and other transcriptional components endogenously present in a nuclear extract of the HER2/*neu* overexpressing breast cancer cell line, SKBR-3. Interestingly, polyamide **3,** which was 3-fold less inhibitory than **1** at inhibiting formation of ESX-TA5 complexes on the 34-bp TA5 promoter probe (IC_{50} of 18 nM *versus* 5 nM), was at least as effective as **1** at inhibiting cell-free transcription off the ~500-bp (R06) HER2/*neu* promoter-driven template. Moreover, the inhibitory activity of polyamide **1,** which was unaffected in EMSA by prebinding of ESX to TA5, was moderately reduced in the cell-free transcription assay by prebinding of nuclear extract to the HER2/*neu* promoter-driven template, as was the transcription inhibitory activity of polyamide **2**. Similar discordances were observed in comparisons of mitoxantrone and Dist as inhibitors of both protein-DNA complex formation and cell-free transcription with the DHFR promoter (11). Possible variables accounting for these discordances in the present study include the multiplicity of endogenous HER2/*neu* promoter binding factors present in the nuclear extract fueling the transcription assay (*versus* the single protein component in the EMSA assay) and potentially different numbers of lower affinity binding sites for each polyamide on the linearized R06 plasmid-containing HER2/*neu* promoter-driven template (*versus* the 34-bp TA5 EMSA probe). To address the potential impact of DNA content (bp) as a discordance-inducing variable between the EMSA and cell-free transcription assay, *r* values were calculated to compare the molar ratios of polyamide to DNA content (Tables I, and II). The degree of difference between the EMSA and transcription assay *r* values for Dist is most notable and without obvious explanation. However, the lower overall *r* values among polyamides tested by transcription assay *versus* their EMSA determined values suggest that differences in total DNA content or polyamide binding sites on the HER2/*neu* promoter-containing plasmid template did not substantially contribute to the discordances noted above.

In summary, polyamides designed to selectively target critical 7-bp elements flanking and overlapping on a singular EBS in the regulatory region of the proximal HER2/*neu* promoter were shown to exhibit high affinity binding to their respective elements and to specifically disrupt binding of a HER2/*neu* promoter EBS candidate, ESX. These Ets-DNA complex inhibiting hairpin polyamides were significantly more potent inhibitors of HER2/*neu* promoter-driven transcription than the natural product Dist, a TATA box minor groove binder, and less effective Ets-DNA complex inhibitor. The differences noted in the HER2/*neu* promoter inhibiting activities of these polyamides is thought to be because of both their respective binding affinities and the choice of EBS flanking elements targeted for polyamide binding. These differences may implicate vulnerable promoter elements for future attempts to repress transcription of the overexpressing HER2/*neu* oncogene. Studies are now underway to evaluate the effectiveness of polyamides as HER2/*neu* transcription inhibitors in whole cell systems.

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